Arabidopsis mpk6-1 mutants and its non-host resistance against *Magnaporthe oryzae*

Thesis submitted to Department of Life Science for the partial fulfilment of the *M.Sc. Degree in Life science*

Under the guidance of

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Submitted by
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CERTIFICATE

This is certified that the thesis entitled **"*Arabidopsis mpk6-1* mutant and its nonhost resistance against *M oryzae*"** which is being submitted by Mrs. Astha, Roll No-413LS2021, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other institution and university for the award of any degree or diploma.

09/05/2015

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DECLARATION

I, Astha hereby declare that, the project report entitled "***Arabidopsis mpk6-1* mutants and its non-host resistance against *Magnaporthe oryzae***" is the original work carried out by me under the supervision of Dr. Binod Bihari Sahu, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge and beliefs the present work has not been presented to any other institution for the award of any other.

Date: ASTHA

Place: NIT, Rourkela

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Date:

Place: Astha

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LIST OF ABBREVIATION

NHR- Nonhost resistance

PRR- Pathogen recognition receptor

MAMPS/PAMPs- Microbial- or Pathogen associated molecular patterns

PTI- PAMPs triggered immunity

ETI- Effectors triggered immunity

HR- Hypersensitive response

MAP-kinase- Mitogen activated protein kinase

ROS- Reactive oxygen Species

PR-Pathogen related

PEN1, PEN2 and PEN3-Penetration gene

ABC -ATP-binding cassette transporter

PDR- Pleiotropic drug resistance

EDS1-Enhanced disease susceptibility 1,

PAD4-phytoalexin-deficient 4

SAG101- senescence-associated gene

SA- salicylic acid

JA -jasmonic acid

NBS- nucleotide-binding site

LRR- leucine-rich repeats

PDA-Potato dextrose agar

ABSTRACT

Rice is one of the most important grain with regard to human nutrition and caloric intake, providing more than one fifth of the calories consumed worldwide by humans. It is the most widely consumed staple food for a large part of the world's human population, especially in Asia. It is an agricultural commodity with the third-highest worldwide production, after sugarcane and maize. A crop failure possess a real threat of starvation. Rice blast, caused by a fungus M. oryzae. It can affect all above ground parts of a rice plant: leaf, collar, node, neck, parts of panicle, and sometimes leaf sheath. Rice blast is one of the most devastating diseases of rice. A leaf blast infection can kill seedlings or plants up to the tillering stage. At later growth stages, a severe leaf blast infection reduces leaf area for grain fill thus reducing grain yield. Leaf blast can kill rice plants at seedling stage and cause yield losses in cases of severe infection. Arabidopsis is a model plant for studying NHR against several plant pathogens. Here, nonhost resistance in *Arabidopsis* is studied against rice blast pathogen *M. oryzae*. The infection in mpk6-1 mutants of Arabidopsis was higher in comparison with wild type Col-0. This was evident from confocal microscopy. Furthermore, on studying the expression of PR1, it can be concluded that the pathogen is hemibiotrophic as PR1 expression was absent.

Keywords: Rice blast, NHR, Pattern-recognition receptors

INTRODUCTION

A wide range of potentially pathogenic microbes are found in the environment. As a result, they have evolved intricate defense mechanisms by which they recognize and defend themselves against a wide array of these disease-causing agents by inducing a set of defense responses that can defeat the invading pathogens. These responses include a hypersensitive response (HR; rapid localized cell death at the site of infection), increased expression of defense related genes [e.g. pathogenesis-related (PR) genes], and the oxidative burst (B.J. et al. (1995); Cutt et al., (1992); Mehdy (1994)). Often, the plant disease resistance described is cultivar or accession specific and is referred to as host resistance. A second resistant, which was shown by an entire plant species to a specific parasite or pathogen is known as nonhost resistance, and is expressed by every plant towards the majority of potentially pathogenic microbes (Groth et al., 1985). A pathogen that cannot cause disease on a nonhost plant is referred to as a nonhost pathogen.

Plants, unlike mammals, lack mobile defender cells and a somatic adaptive immune system. Instead, they rely on the innate immunity of each cell and on systemic signals emanating from infection sites (Danghl et al., & Ausubel (2005), Chisholm et al.,). Previously reviewed the disease resistance (R) protein diversity, polymorphism at R loci in wild plants and lack thereof in crops, and the suite of cellular responses that follow R protein activation (Dangl et al., and Briaen et al., 1998). It hypothesized that many plant R proteins might be activated indirectly by pathogen-encoded effectors, and not by direct recognition.

This ‘guard hypothesis’ implies that R proteins indirectly recognize pathogen effectors by monitoring the integrity of host cellular targets of effector action (Dangle et al., year). The concept that R proteins recognize ‘pathogen- induced modified self’ is similar to the recognition of ‘modified self’ in ‘danger signal’ models of the (Matzinger 2002) mammalian immune system.

It is now clear that there are two branches of the plant immune system. One uses transmembrane pattern recognition receptors (PRRs) that respond to slowly evolving microbial- or pathogen-associated molecular patterns (MAMPS or PAMPs), such as flagellin (Zipfel et al., 2005). The second acts largely inside the cell, using the polymorphic NB-LRR protein products encoded by most R genes (Dangl et al.). They are named after their characteristic nucleotide binding (NB) and leucine rich repeat (LRR) domains. NB-LRR proteins are broadly related to animal CATERPILLER/NOD/NLR proteins (Tinge et al., 2005) and STAND ATPases (Liepe et al., 2004). Pathogen effectors from diverse kingdoms are recognized by NB-LRR proteins, and activate similar defence responses. NBLRR- mediated disease resistance is effective against pathogens that can grow only on living host tissue (obligate biotrophs), or hemibiotrophic pathogens, but not against pathogens that kill host tissue during colonization (necrotrophs) (Glazebrook et al., 2005).

Pathogens of all lifestyle classes (color coded and labeled) express PAMPs and MAMPs as they colonize plants (shapes are color coded to the pathogens). Plants perceive these via extracellular PRRs and initiate PRR-mediated immunity (PTI; step 1). Pathogens deliver virulence effectors to both the plant cell apoplast to block PAMP/MAMP perception (not shown) and to the plant cell interior (step 2). These effectors are addressed to specific

subcellular locations where they can suppress PTI and facilitate virulence (step 3). Intracellular NLR receptors can sense effectors in three principal ways: first, by direct receptor ligand interaction (step 4a); second, by sensing effector-mediated alteration in a decoy protein that structurally mimics an effector target, but has no other function in the plant cell (step 4b); and third, by sensing effector-mediated alteration of a host virulence target, like the cytosolic domain of a PRR (step 4c). It is not yet clear whether each of these activation modes proceeds by the same molecular mechanism, nor is it clear how, or where, each results in NLR-dependent effector-triggered immunity (ETI). [Modified from by Sarah R. Grant]

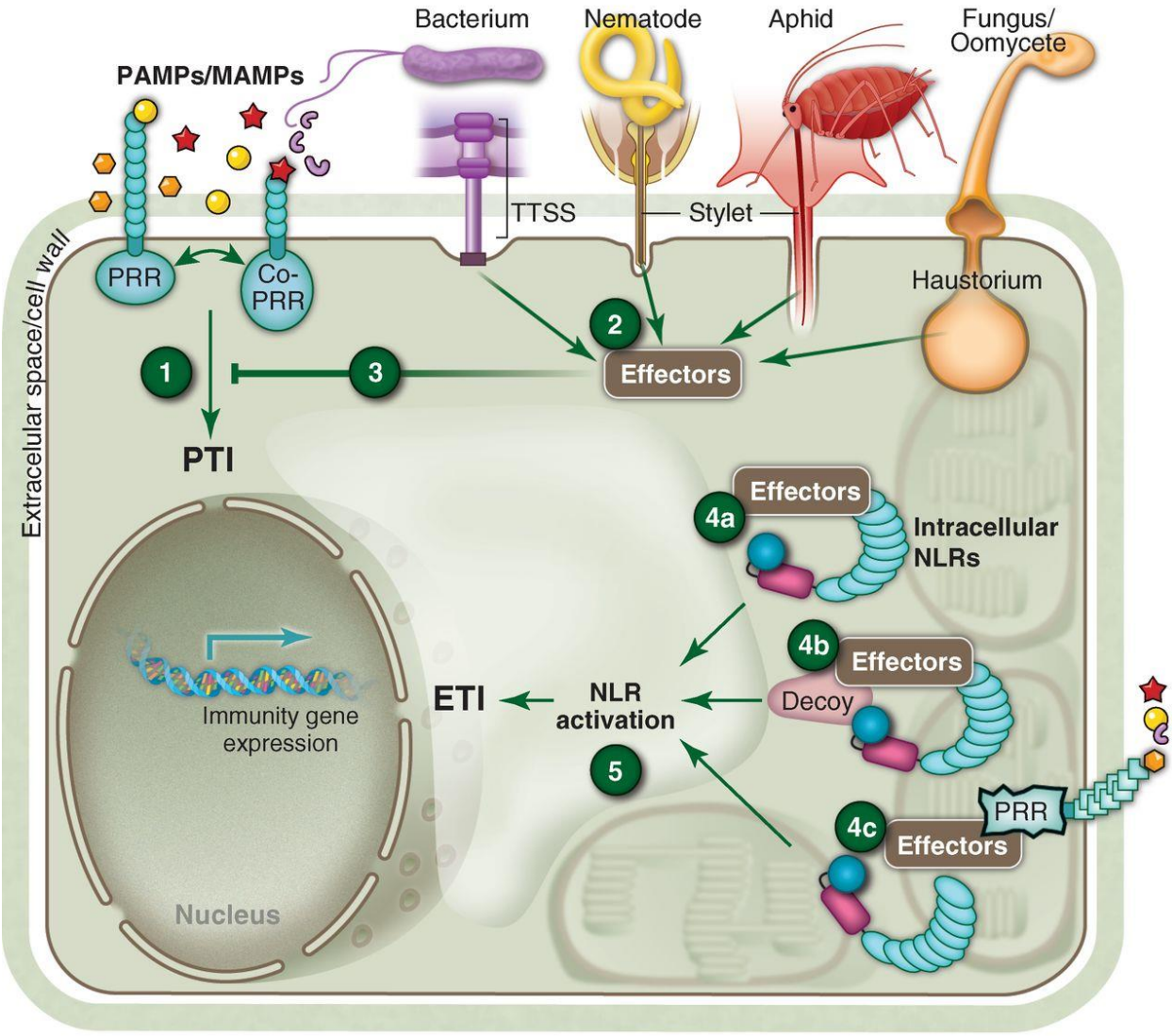


Figure 1: Schematic of the plant immune system.

Rice blast disease was caused by fungus *Magnaporthe oryzae*, is a devastating disease of rice worldwide. Magnaporthe oryzae is a filamentous ascomycete fungus. The importance of rice is underscored by the fact that roughly one-half of the world's populace depends on rice for its essential caloric admission (Khush, 2005). All foliar tissues are liable to infection; however, infection of the panicle leads to complete loss of grain. Losses of 10%–30% are typical, although regional epidemics can be devastating. In spite of this the fungus is highly amenable to genetic and molecular genetic manipulation (Jeon et al., 2007; Talbot, 2003;

Valent and Chumley, 1991). Consequently, as a result of its agronomic importance and tractability, *M. oryzae* has emerged as a seminal model in the study of host–fungal pathogen interactions (Dean et al., 2005). Although host resistance is the most economically viable and environmentally sound practice to manage the disease, the fungus overcomes blast resistance quickly, and cultivars typically become ineffective within 2–3 years (Ou, 1980; Zeigler et al., 1994). Magnaporthe oryzae is part of a species complex that can cause disease on a variety of grasses and related species, including crops such as barley, wheat and millet (Couch et al., 2005). New wheat strains have emerged in South America, elevating concerns that the fungus poses a serious threat to global wheat production (Urashima et al., 1993).

The mechanisms of Rice resistance to blast mechanism has been studied extensively and the rice-*M. oryzae* pathosystem has become a model in plant-microbe interaction studies(Koga 2001 and Ebbole 2007) but the mechanisms of non-host resistance to rice-blast was not well understood (Okawa and Ishiawaka 2013). Current studies demonstrate that Arabidopsis NHR to nonadapted biotrophic powdery mildews has two successive multicomponent defense layers: penetration and post-penetration resistance. We have found that *PEN2, PMR5, AGB1,* and *MLO2* are involved in both penetration and post-penetration resistance to M. oryzae in A. thaliana (Maeda et al., 2009. 2010 and Nakao et al., in 2011).

Table 1: ***The table represents the ranked list of fungi as voted for by plant mycologists associated with the journal Molecular Plant Pathology***.

Rank	**Fungal pathogen**	**Author of fungal description**
1	*Magnaporthe oryzae*	Ralph Dean
2	*Botrytis cinerea*	Jan A. L. van Kan
3	*Puccinia spp.*	Zacharias A. Pretorius
4	*Fusarium graminearum*	Kim Hammond-Kosack
5	*Fusarium oxysporum*	Antonio Di Pietro
6	*Blumeria graminis*	Pietro Spanu
7	*Mycosphaerella graminicola*	Jason J. Rudd
8	*Colletotrichum spp.*	Marty Dickman
9	*Ustilago maydis*	Regine Kahmann
10	*Melampsora lini*	Jeff Ellis

REVIEW OF LITERATURE

Plants are now exposed to a large number of pathogens and as a result they evolved in intricate defense mechanism in order to recognize and defend themselves against the large variety of array of these disease causing organism. Disease causing agents by inducing a set of defense responses that can defeat the invading pathogens. These responses include a hypersensitive response (HR; rapid localized cell death at the site of infection), increased expression of defense related genes [e.g. pathogenesis-related (PR) genes], and the oxidative burst (B.J. et al., 1995 and Cutt et al., 1992). Often, the plant disease resistance is cultivar or accession specific and is referred to as host resistance. A second type of resistance that is not well understood, provides resistance against pathogens throughout all members of a plant species. This type of resistance is referred to as nonhost resistance (B.J. et al., 1995 and Heath et al., 1987). Nonhost resistance which are shown by an entire plant species to a specific or pathogen or parasite, is the most common and durable form of plant disease resistance (Heath et al., 2000). A pathogen that cannot cause disease on a nonhost plant is referred to as a nonhost pathogen. Host resistance is often governed by single resistance (R) genes, the products of which directly or indirectly interact with the specific elicitors produced by the avirulence (avr) genes of pathogens (Flor 1971 and Hammond-Kosack et al., 1997).

Plant defense signaling

Several plant signaling components are involved during the induction of plant defense. An invading pathogen has to bypass many of these signaling components to cause disease successfully in plants. For example, the plant hormone ethylene is an important signaling component during plant–pathogen interactions. Ethylene perception is often required for basal resistance against pathogens and it can also induce disease resistance in plants. An ethylene-insensitive tobacco has been shown to lack nonhost resistance against several soil-borne fungi (Knoester et al., 1998). Transgenic tobacco plants expressing the *Arabidopsis etr1-1* gene (which causes loss of ethylene perception) were unable to support induction of basic PR genes upon tobacco mosaic virus (TMV) infection, and developed spontaneous stem necrosis during growth in soil. The stem necrosis was attributed to fungal infections by *Pythium sylvaticum*, *Pythium splendens*, other uncharacterized *Pythium* spp., *Rhizopus* spp. and *Chalara elegans* (Knoester et al., 1998). None of these soil-borne fungi infects the wild-type tobacco plants, indicating that tobacco is a nonhost for these fungi and that ethylene signaling might play a role in nonhost disease resistance. A recent report suggests that the Arabidopsis ethylene-insensitive mutants *etr1-1* and *ein2-1* exhibit increased susceptibility to several Arabidopsis pathogens (Geraats et al., 2003). However, the *Arabidopsis etr1-1* mutant has not been reported to lack nonhost pathogen resistance. Thus, the requirement of ethylene perception for nonhost resistance could be plant species specific.

Salicylic acid is one of the key signaling molecules that activate plant defense responses against invading pathogens (Dempsey et al., 1999). Recently, salicylic acid has been implicated in playing a role in nonhost resistance. Arabidopsis is a nonhost for cowpea rust fungus (Uromyces vignae) and hence restricts the growth of this fungus. The Arabidopsis mutant sid2, defective in an enzyme that synthesizes salicylic acid, and *Arabidopsis* NahG plants (which express salicylate hydroxylase that can degrade salicylic acid) support growth of U. vignae

indicating that the salicylic acid pathway is required for nonhost resistance against the rust fungus in Arabidopsis (Mellersh et al., 1997). *Arabidopsis* NahG plants are also susceptible to *Pseudomonas syringae* pv. *phaseolicola*, a pathogen normally unable to infect Arabidopsis (Lu et al., 2001). The loss of nonhost resistance of *Arabidopsis* NahG to *P. syringae* pv. *phaseolicola* has recently been shown to be the result of the presence of catechol, a degradation product of salicylic acid, and not to the lack of salicylic acid itself (Glazebrook et al., 2003). However, the susceptibility of the sid2 mutant to *U. vignae* suggests that salicylic acid synthesis is still required and might play a key role during nonhost disease resistance.

Wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK) have been previously implicated as signaling components of plant defense reactions (Zhang et al., 2001).

WIPK and SIPK in *Nicotiana benthamiana* compromises nonhost resistance against *Pseudomonas cichorii* by allowing multiplication and growth of this nonhost pathogen (Sharma et al., 2003). It is interesting to note that the silencing of either WIPK or SIPK did not alter the HR mediated by the incompatible pathogen *P. cichorii* on the nonhost *N. benthamiana*. It is intriguing that the avirulent pathogen could multiply up to 20-fold more in the WIPKand SIPK-silenced plants, compared to the control, in the presence of an HR. It is possible that there was a slight delay or reduction of the HR in the WIPK- and SIPKsilenced plants, but this was not detectable by the naked eye. Silencing of WIPK and SIPK also did not affect the HR mediated by INF1 (an elicitor from *Phytophthora infestans* that produces HR when inoculated on wildtype *N. benthamiana*, a nonhost for *P. infestans*) on *N. benthamiana* (Sharma et al., 2003). Heat-shock proteins (Hsps) are highly conserved proteins that are induced during various forms of environmental stress. Kanzaki et al. recently showed that *N. benthamiana* Hsp90, a cytosolic protein, interacts with SIPK in a yeast two-hybrid system (Kanzaki et al., 2003).

In many cases, nonhost resistance against fungal pathogens is associated with the penetration process. Arabidopsis pen (penetration) mutants were identified by screening for mutants that showed increased penetration of the nonhost fungal pathogen *Blumeria graminis* f. sp. *hordei*, which causes powdery mildew in barley (Collins et al., 2003). Mutations in the PEN1 and PEN2 genes reduced the ability of the plants to arrest conidia of *B. graminis* f. sp. hordei to , 20% of that of wild-type plants (Thordal-Christensen 2003). Map-based cloning of *PEN1* revealed that it encodes syntaxin and that it might play a crucial role in papilla-related vesicle trafficking in the plasma membrane (Collins et al., 2003). Syntaxins are members of the SNARE super family of proteins that mediate membrane-fusion events. The pen2 mutant shows alteration of cell-wall-related structure suggesting that the cell wall structures play an important role as physical barriers against fungal infections (Thordal-Christensen 2003). Similar screening with the *B. graminis* f. sp. *hordei* pathogen on the host plant barley isolated two mutants, ror1 and ror2 (required for MLO-specified resistance), which enhance penetration of *B. graminis* f. sp. *hordei* (Freialdenhoven et al., 1996). Interestingly, *ROR2* gene is a functional homolog of PEN1 gene (Collins et al., 2003). These results provide a mechanistic link between non-host and basal penetration resistance.

Magnaporthe oryzae

Magnaporthe oryzae (anamorph: *Pyricularia grisea*) is a plant pathogen isolated from rice and other rice field weeds also called as rice blast fungus. It influences all the phases of the plant with severe damage amid the seedling stage. This fungi produces spores that can be dispersed by wind and sprinkling precipitation. The spores can spend the winter in rice grains and rice stubble and can infect new crops the accompanying year. Infection is more probable over long stretches of downpour or high stickiness. There are known strains of rice resistant to this infection that may be useful for it control. *Magnaporthe oryzae* is the most essential rice pathogen overall known to occur in 85 nations. Consistently, the loss in crops because of rice blast could sustain 60 million individuals. The *Magnaporthe oryzae* genome was release as a component of the Magnaporthe similar database, it as size of 41.03 Mb and encodes around 12,593 protein-coding genes. The fungus making the strongest appearance was *Magnaporthe oryzae*, the economic importance of *M. oryzae*; with over one-half of the world's population relying on rice as the main source of calories, this pathogen can have devastating effects; however, many also highlighted how this pathogen has developed into a model system for the study of plant–pathogen interactions. In this The fungus making the strongest appearance was Magnaporthe oryzae, the economic importance of *M. oryzae*; with over one-half of the world's population relying on rice as the main source of calories, this pathogen can have devastating effects; however, many also highlighted how this pathogen has developed into a model system for the study of plant–pathogen interactions. Here figure 1 represents the infection cycle of rice blast fungus. Infection cycle of the rice blast fungus. Conidia produced from lesions are splashed into new plants where they attach firmly and germinate within a few hours. Subsequently, the germ tube ceases polar growth, the tip swells and, by 12 h, forms a highly melanized dome-shaped structure, the appressorium. Typically within 24 h, turgor pressure increases within the appressorium, forcing a penetration peg into the underlying tissues. Eye-shaped necrotic lesions do not appear until several days after infection, from which, under appropriate conditions, conidiophores emerge bearing conidia to re-initiate the infection cycle.

Figure 2: Disease resulting from the infection of rice and wheat with Magnaporthe oryzae. Classical symptoms of panicle blast on rice, although the fungus can cause disease on all foliar tissues.

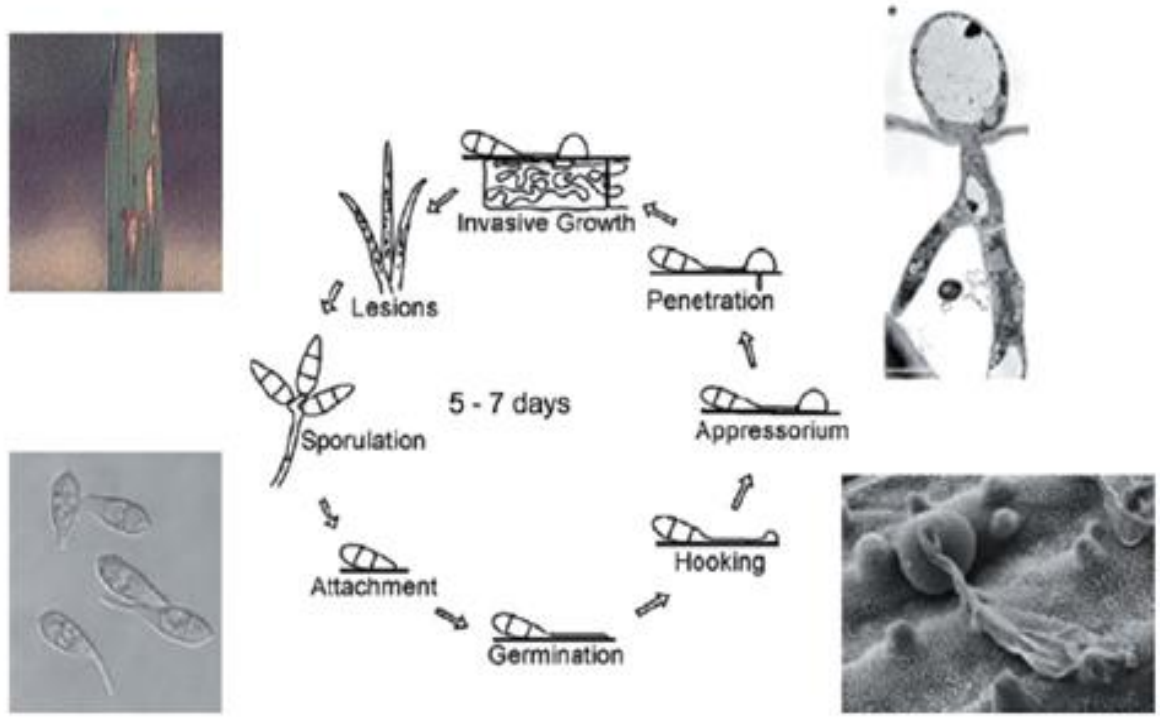


Figure 3: Figure represents the Infection cycle of the rice blast fungus.

Non host resistance

In nature, majority of plant species are resistant to most pests and pathogen species. This form of disease resistance is known as non-host resistance (NHR). Non-host resistance is the most common, durable and non-specific type of resistance observed in plant-pathogen interactions, making this type of resistance of great interest for agriculture (Heath, 2000). Non-host resistance is defined as a full resistance at the species or genus level (Kamoun et al., 1999b). It is thought to be genetically complex, involving preformed and inducible defences (Freialdenhoven et al., 2005). Non-host resistance is considered to be durable in the field (Vleeshouwers et al., 2000). Kamoun and associates (1998) reported that durable and stable resistance responses may have evolved in non-host plants through the accumulation of an arsenal of R-genes. Nonhost resistance is presumed to be a complex, multi-component form of resistance, including both constitutive and inducible defences. Non-host resistance may also result from pathogen species being poorly adapted to the basic physiology or growth habit of the plant species (Kamoun et al., 1999). A major component of non-host resistance is penetration resistance (Zimmerli et al., 2004). Notably, the non-host resistance in Arabidopsis to the wheat powdery mildew appears to consist primarily of *EDS1* (Enhanced Disease Susceptibility 1) (Yun et al., 2003; Zimmerli et al., 2004). The salysilic acid (SA) signal transduction pathway plays an important role in defense responses initiated by R-genes (Glazebrook, 2001); however, its contribution to nonhost resistance is less clear (Parker et al., 1996).

Host Plant Resistance (HPR)

"Those characters that enable a plant to avoid, tolerate or recover from attacks of insects under conditions that would cause greater injury to other plants of the same species" (Painter, R.H.,

1951). “Those heritable characteristics possessed by the plant which influence the ultimate degree of damage done by the insect” (Maxwell, F.G., 1972). Host plant resistance can be of two types, Ecological resistance or Pseudo resistance which occurs in susceptible host plants due to environmental conditions. And second is Genetic resistance which is further classified based on their biotype reactions, number of genes, evolutionary concept and population/line concept.

Type I nonhost resistance

A model was proposed for the mechanism of non-HR-mediated type I nonhost resistance (Figure 3). After a pathogen lands on a nonhost plant, it tries to enter the host tissue in search of nutrition. The first obstacle that the pathogen will face is preformed plant barriers (passive defense mechanisms), such as cell walls, antimicrobial compounds and other secondary metabolites (Thordal-Christensen, 2003; Osbourn 1996). The second obstacle the pathogen will face is the inducible plant defense responses (active defense mechanisms). Plants recognize general elicitors from pathogens in a nonspecific manner to activate defense responses. For example flagellin, a general elicitor protein from bacterial flagella, activates defense responses through a MAP kinase cascade in *Arabidopsis* (Matzinger 2003). Plants can also recognize pathogen surface molecules, also referred to as pathogen-associated molecular patterns (PAMPs), to induce innate immunity (Nurnberger et al., 2002; Gomez-Gomez et al., 2002). PAMPs are shared among members of a pathogen group and are known to induce innate immunity in both plants and animals. Some of the plant defense responses that are induced because of general elicitors and PAMPs include cell wall thickening, cell wall lignification, accumulation of phenolics, production of saponins, and production of phytoalexins, papilla formation and induction of PR genes (Heath, M.C. (1997) McLusky et al., 1999).

During type I nonhost resistance, the pathogen will not be able to get past the first or the second obstacle, and the multiplication and penetration into the plant cell will be completely arrested. Even though the plant looks normal (without any visible symptoms) during the type I nonhost resistance, several molecular changes might be happening.

Type II nonhost resistance

The most common of nonhost resistance is the type II that produces a nonhost HR. Type II nonhost resistance is phenotypically more similar to an incompatible gene-for-gene interaction and is a more sophisticated plant defense mechanism than type I nonhost resistance.

Some pathogens can conquer early obstacles by producing detoxifying enzymes to overcome the toxic effect of preformed antimicrobial plant secondary metabolites. The next obstacle the pathogen will face is the plant cellular defense surveillance mechanism. Plants have evolved to recognize certain pathogen elicitors, either in the plant cytoplasm or at the plant cell membrane, which trigger a defense mechanism that will often lead to HR. Such pathogen elicitors that can be recognized by plants to activate defense responses are called avirulence (Avr) proteins. Avr proteins when not recognized by plants can enhance the virulence of pathogens (Shan et al., 2000).

Once a pathogen can overcome preformed and general elicitor induced barriers, fungal and oomycete pathogens can directly penetrate a plant cell whereas most plant bacterial pathogens inject virulence and avirulence proteins into the plant cell through a hrp gene-encoded type III secretion system (TTSS) (Hutcheson 2001) (Fig. 3). For fungal and oomycete pathogens, the

extracellular proteins on the hyphae or secreted proteins serve as elicitors whereas the injected avirulence proteins serve as elicitors for bacterial pathogens. An elicitor(s) will be recognized by the plant surveillance system and a defense reaction leading to HR will be activated. This will prevent the further spread of the pathogen from the infected cell.

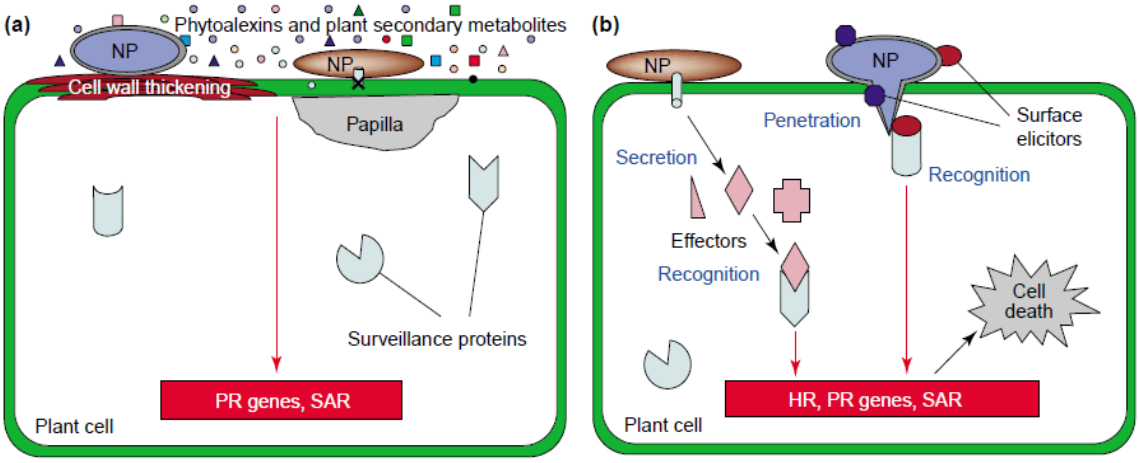


Figure 4: Type 1 and type 2 nonhost resistance

Table 2: Examples of type I and type II nonhost resistance genes			
Pathogen	Strain or isolate	Nonhost plant(s)	Visible symptoms
Type I nonhost resistance			
Pseudomonas syringae pv. phaseolicola	NPS3121	*Arabidopsis*	None
P. s. pv. phaseolicola (at 30.8C)	S-2	Nicotiana tobacum	None
P. s. pv. syringae	B-76	Arabidopsis	None
P. s. pv. Savastanoi	213-3 (IAA2)	Arabidopsis	None
P. s. pv. Delphinii	PDDCC529	Arabidopsis	None
P. s. pv. morsprunorum	B60-1	Arabidopsis	None
P. s. pv. Atrofaciens	B143	Arabidopsis	None
P. s. pv. Coronafaciens	B142	Arabidopsis	None

Xanthomas campestris pv. Campestris	8004	Nicotiana benthamiana	None
Gaeumannomyces graminis var.tritici	T5	Avena strigosa	None
Puccinia recondita f. sp.tritici	TBR1	Oat	None
Puccinia graminis f. sp.tritici	ANZ	Oat	None
Phytophthora infestans	88069	N. alata cv. Lime green	None
P. infestans	88069	N. clevelandii	None
P. infestans	88069	N. tabacum cv. xanthi	None
Type II nonhost resistance			
Pseudomonas syringae pv.maculicola	m2	Nicotiana benthamiana	HR
P. s. pv. tomato	DC3000	N. tabacum	HR
P. s. pv. phaseolicola	NPS3121	N. tabacum	HR
P. s. pv. Glycinea	PG4180	N. tabacum	HR
P. s. pv. pisi	ATCC#11055	N. tabacum	HR
P. s. pv. syringae	61	N. tabacum	HR
P. cichorii	83-1	Arabidopsis	HR
Xanthomonas axinopodis pv. vesicatoria	82-8	Nicotiana benthamiana	HR
X. campestris pv. glycines	8ra	Pepper, tomato	HR
X. citri	3213	Cotton, bean	HR
Erwinia rubrifaciens		N. tabacum	HR
Alternaria brassicicola	MUCL20297	Arabidopsis	HR
Blumeria graminis f. sp. tritici	bgtA95	Barley	HR
Phytophthora infestans		Arabidopsis	HR
P. infestans	88069	N. benthamiana, N. rustica, parsley	HR
P. sojae			HR
Fusarium solani f. sp. phaseoli	W-8	Pea	HR

Arabidopsis thaliana:

A model plant that is nonhost for the *Magnaporthe oryzae*

Arabidopsis thaliana is a small flowering plant commonly used as a model plant in plant biology. *Arabidopsis* is native to Eurasia. It is a member of the mustard (Brassicaceae) family, which includes cultivated species such as radish and cabbage. Arabidopsis thaliana was discovered by Johannes Thal (hence, thaliana) in the Harz Mountains in the sixteenth century, though he called it *Pilosella siliquosa*. The potential for Arabidopsis thaliana as a model organism was first summarized by F. Laibach in 1943 for genetics.

Ecotypes and Geographic Distribution

More than 750 natural accessions of Arabidopsis thaliana have been collected from all over the world and the two major seed stock centers, ABRC and NASC, where it is available. These accessions are quite variable in terms of form and development (e.g. leaf shape, hairiness) and physiology (disease resistance, flowering time). Around the world various researcher are using these differences in natural accessions in order to uncover the complex genetic interactions such as those underlying plant responses to environment and evolution of morphological traits.

- *Arabidopsis* is advantageous plant for basic research in genetics and molecular biology.
- Approximately 115 Mb of the 125 Mb genome has been sequenced and annotated (Nature, 408:796-815; 2000).
- Extensive genetic and physical maps of all 5 chromosomes are available.
- The life cycle is short--about 6 weeks from germination to seed maturation.
- Seed production is prolific and the plant is easily cultivated in restricted space.
- Transformation is efficient utilizing *Agrobacterium tumefaciens*.
- A large number of mutant lines and genomic resources is available.
- *A. thaliana* is studied by a multinational research community in academia, government and indzus. Such advantages have made *Arabidopsis* a model organism for studies of the cellular and molecular biology of flowering plants. The Arabidopsis Information Resource (TAIR) collects and makes available the information arising from these effortstry.

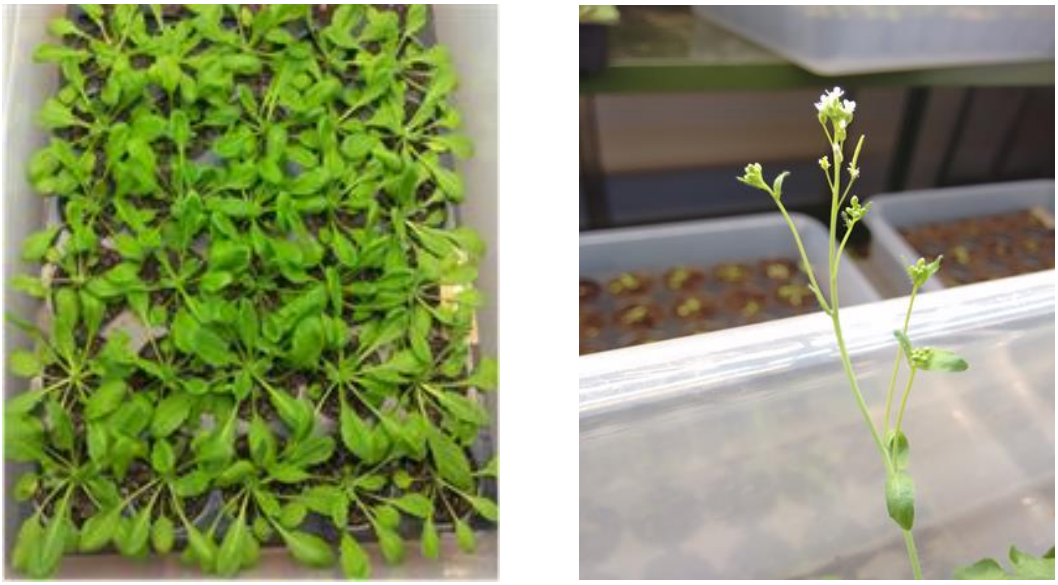


Figure 5: Arabidopsis thaliana

Mpk6-1: An Arabidopsis mutant

Mitogen-activated protein kinase (MAPK) cascades are signal transduction modules that are highly conserved in eukaryotes (Zhang et al., 2006). A MAPK module consists of three kinases: a MPKKK, a MPKK, and a MPK. All the three types of MPK participate in downstream process and activates the downstream targets by phosphorylation. The last kinase of the module, a MPK, is able to phosphorylate several substrates, including transcription factors, to regulate gene expression (Andreasson and Ellis, 2009). MAPKs are known regulators of biotic and abiotic stress responses, hormone perception, and developmental programmes (Colcombet and Hirt, 2008; Suarez-Rodriguez et al., 2010).

We found that the mpk6 mutation caused altered embryo development giving rise to three seed phenotypes that, post-germination, correlated with alterations in root architecture. In the smaller seed class, mutant seedlings failed to develop the primary root, possibly as a result of an earlier defect in the division of the hypophysis cell during embryo development, but they had the capacity to develop adventitious roots to complete their life cycle. In the larger class, the MPK6 loss of function did not cause any evident alteration in seed morphology, but the embryo and the mature seed were bigger than the wild type. Seedlings developed from these bigger seeds were characterized by a primary root longer than that of the wild type, accompanied by significantly increased lateral root initiation and more and longer root hairs. Apparently, the increment in primary root growth resulted from an enhanced cell production and cell elongation. Our data demonstrated that MPK6 plays an important role during embryo development and acts as a repressor of primary and lateral root development.

The Arabidopsis genome encodes 20 different MPKs (MAPK Group, 2002), from which MPK3, MPK4, and MPK6 play important roles both in stress and developmental responses (Colcombet and Hirt, 2008). In particular, MPK6 has been found to participate in bacterial and fungal resistance (Nuhse et al., 2000; Asai et al., 2002; Menke et al., 2004; Wan et al., 2004; Zhou et al., 2004; Zhang et al., 2007), in mutualistic interactions (Vadassery et al.,

2009), in priming of stress (Beckers et al., 2009), and in regulation of plant architecture (Bush and Krysan, 2007; Müller et al., 2010).

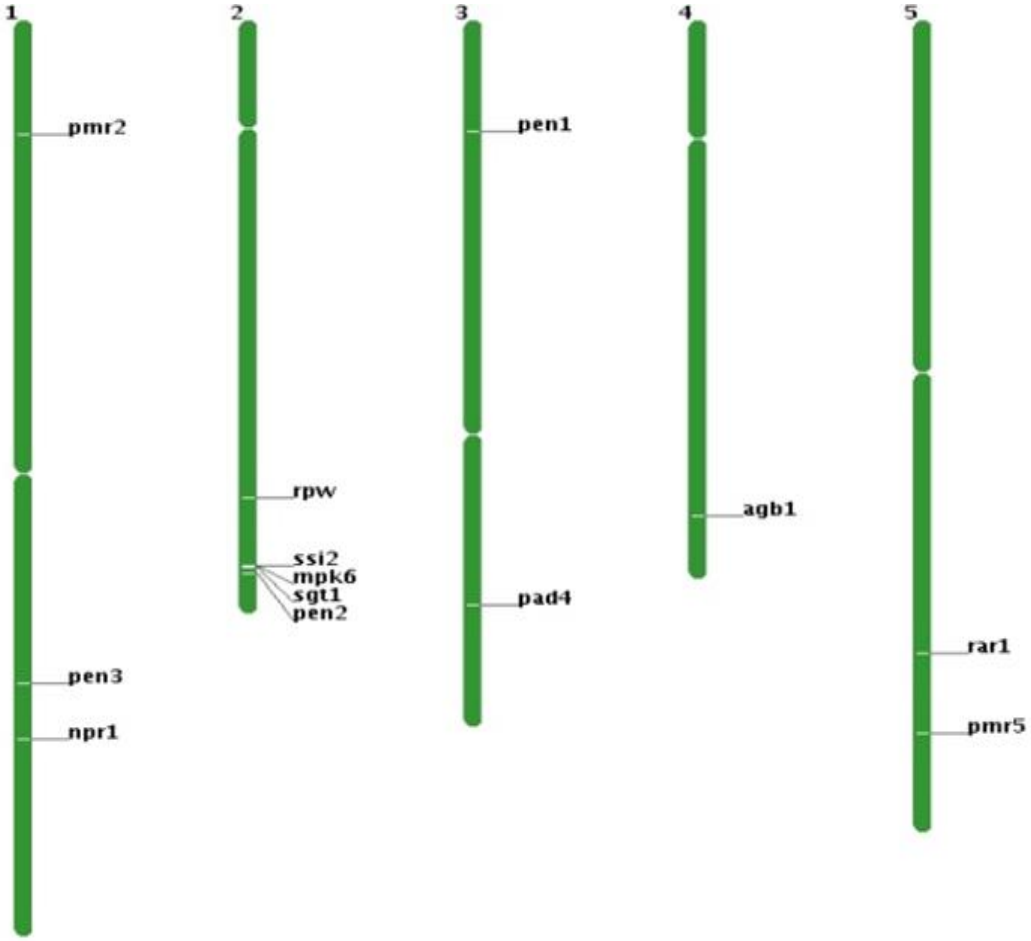


Figure 6: Molecular map of nonhost resistance genes

Functional redundancy is common among MAPKs. Particularly, MPK3 and MPK6 participate in biotic and abiotic stress resistance as well as in developmental processes (Lee and Ellis, 2007; Hord et al., 2008; Lampard et al., 2009; Liu et al., 2010). MPK4/MPK6 and even MPK3/MPK4/MPK6 have been shown to act redundantly in osmotic, touch, wounding, and defence responses (Ichimura et al., 2000; Droillard et al., 2004; Meszaros et al., 2006; Brader et al., 2007). MPKs are proposed to act through common downstream targets and upstream activators (Feilner et al., 2005; Merkouropoulos et al., 2008; Andreasson and Ellis, 2009; Popescu et al., 2009), but the interaction of these pathways is poorly understood. The MPK6 loss-of-function mutant displays alterations in the embryo and early root development, indicating that, at least for these processes, the function of this kinase cannot be substituted by any other MPK (Bush and Krysan, 2007; Müller et al., 2010; Wang et al., 2010).

The observed mpk6 phenotypes display variable penetrance, probably influenced by the growth conditions. Additionally, mutations in the MPK6 gene have been linked to protrusion of the embryo detected in about 7% of the seeds from an mpk6 homozygous population (Bush and Krysan, 2007).

OBJECTIVES

1. To study the phenotype of the wild type Col-0 and mutant mpk 6-1 infected by M. oryzae
2. To check the DNA integrity after infection
3. To assess the involvement of dense pathway genes during infection with M. oryzae

MATERIALS & METHODS

1. Sample preparation

1.1.Plant material: *Arabidopsis* seeds were collected from "Nottingham Arabidopsis Stock center" (NASC) and stored at 4°C. The Arabidopsis accession code was Col-O. We used the mutant mpk 6-1. Then, seed samples were soaked in distilled water in a 1.5 ml eppendorf tube overnight (that is needed for good germination and breaking the dormancy).

1.2. Soil preparation: Agropeat soil were mixed with vermiculite in the ratio 1:5, mixed it well. Then fill the pots with the mixture of soil. Seeds were then soaked in the mixture of soil then cover it with thin plastic film so that humidity was maintained. Light and temperature should be maintained. Light should be maintained at 12*7(hour/ days) and Temperature of plant growth chamber should be maintained at 21°C. After 3 days, uncovered the tray and seedlings formation occurred. For the better growth of plant water and fertilizer were given to the plant alternately. After 11 days plantlet were transplanted individually in the pot. For maintaining the humidity the tray was covered for three days.

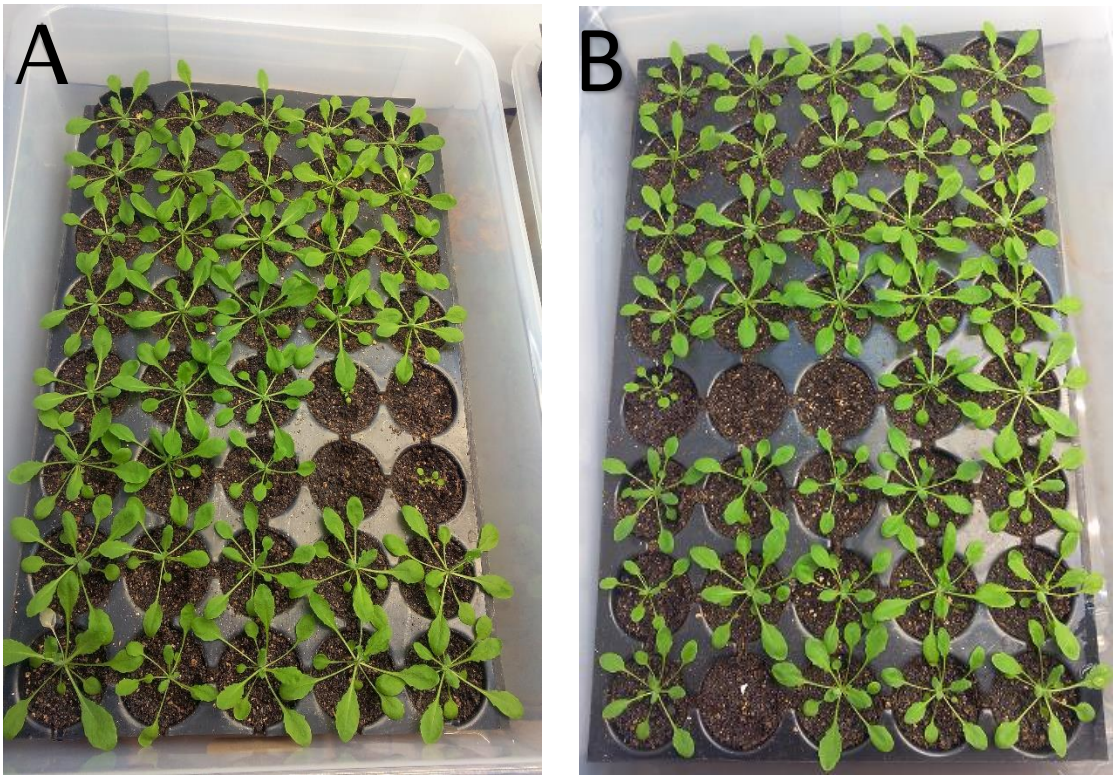


Figure7: Growth of Arabidopsis (A) wild type (Col-O) and (B) mutant plant (mpk 6-1)

1.3. Fertilizer preparation:

Chemical	Amount
Ammonium nitrate	6.516g in 1000ml
Potassium chloride	7.6249g in 1000ml
Disodium hydrogen phosphate	1.824g in 1000 ml

100ml was taken from each stock solution and added water to maintained volume 600ml and pouring each tray.

2. Fungal material:

Fungus Magnaporthe oryzae was collected from National Center for Plant Genomic Research (NCPGR) of strain Himalayan isolate. Magnaporthe oryzae isolate was incubated on oatmeal agar media and potato dextrose agar (PDA) media in petridish at 25°C. Then, the inoculum was prepared. In order to inoculate M. oryzae, spores were diluted 10 µl droplets (10^5 spores/ml). In the culture plates water was added, shake the plate, then transfer the spores in the falcon tube**.**

2.1.Oat meal agar media

For 100ml media, 6.00gm of oat meal powder (HIMEDIA) and 1.25 gm of agar (HIMEDIA) was dissolved in 100ml of distilled water and pH of 7.2±0.2 was maintained. Then autoclaved at 121°C at 15 lbs pressure for 15-20 minutes for sterilization. Then 100 µl of streptomycin was added in it before pouring in petridish.

2.2.Potato dextrose agar

In 100ml PDA media, 1.3 gm of potato dextrose agar (HIMEDIA) and 1 gm of agar(HIMEDIA) was mixed in 100 ml of distilled water. Then autoclaved at 121°C at 15 lbs pressure for 15-20 minutes for sterilization. Then 100 µl of streptomycin was added in it before pouring in petridish. Then about 25µl of media was poured in each petridish.

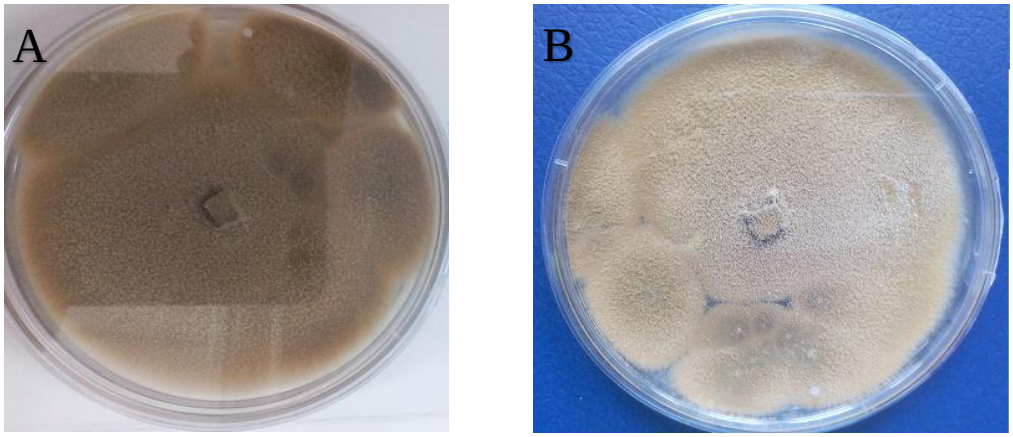


Figure 8: Growth of Magnaporthe oryzae A) Growth on Potato dextrose agar B) Growth on Oat meal agar media

3. Leaf infection:

For fungal infection, autoclavable petriplate (150mm* 25mm) was taken, filter paper was placed on the petriplate. Plant leaf of 3-5 weeks was taken and arranged in a triplet. In the first triplet, water was placed on the surface of the leaf. Then, 10µl of spores was placed on all other triplet leaves. The inoculated leaf sample was then placed in 25°C and sealed it with parafilm so that humidity was maintained. Then, after 1 day infection, phenotype was observed.

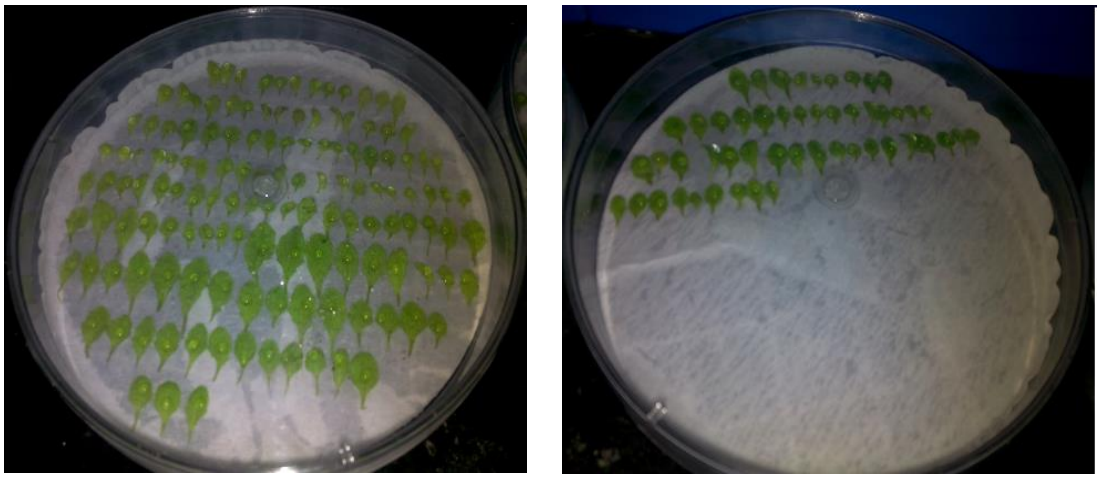


Figure 9: Leaf infection by Magnaporthe oryzae spore (a) Col-O (b) mpk 6-1

4. Staining

For analyze the infection we take 1 day infected leaves for staining. Trypan blue stained the dead cell and aniline blue stained the callose deposition.

Fixing solution

Leaf samples were dipped in fixing samples (60% methanol, 30%chloroform and 10% acetic acid) and left for overnight.

4.1 Trypan blue

- Fixed sample were rehydrated through decreasing ethanol (100%, 80%, 70% and 50% ethanol).
- Samples then were stained in 0.05% trypan blue in distilled water overnight.
- De-staining was done in distilled water in next day.
- The leaves then were mounted in 30% glycerol on glass slides.

4.2 Trypan-aniline blue combination (dual staining)

- Leaf samples were re hydrated through decreasing ethanol (100%, 80%, 70% and 50% ethanol).
- Samples were soaked in 0.05% trypan blue for overnight and then soaked in 0.05%aniline blue in 150mM KH_2PO_4, pH9.5 for 3-4 hr.
- The leaves then were distained in 150mM KH_2PO_4 and 2 to 3 times for 15 minutes and mounted on glass slides.

For visualization the stained cell, slides are observed under fluorescence (trypan blue staining slides) and confocal microscope (dual staining) and took the images.

The Trypan blue stained leaf sample was then observed under Fluorescence microscopy and the combination of trypan- aniline blue or dual stained sample was observed under confocal microscopy.

4.3 Microscopy

4.3.1 Fluorescence microscopy

A fluorescence microscope is an optical microscope that uses fluorescence and phosphorescence instead of reflection and absorption to study properties of organic or inorganic substances. The "fluorescence microscope" refers to microscope that uses fluorescence to generate an image, whether it is a simpler set up like an epifluorescence microscope, which uses optical sectioning to get better resolution of the fluorescent image.

The specimen is illuminated with light of a specific wavelength which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e., of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. Typical components of a fluorescence microscope are a light source (xenon arc lamp or mercury-vapor lamp are common; more advanced forms are high-power LEDs and lasers), the excitation filter, the dichroic mirror (or dichroic beamsplitter), and the emission filter (figure). The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen. In this manner, the distribution of a single fluorophore (color) is imaged at a time. Multi-color images of several types of fluorophores must be composed by combining several single-color images. These microscopes are widely used in biology and are the basis for more advanced microscope designs, such as the confocal microscope and the total internal reflection fluorescence microscope (TIRF).

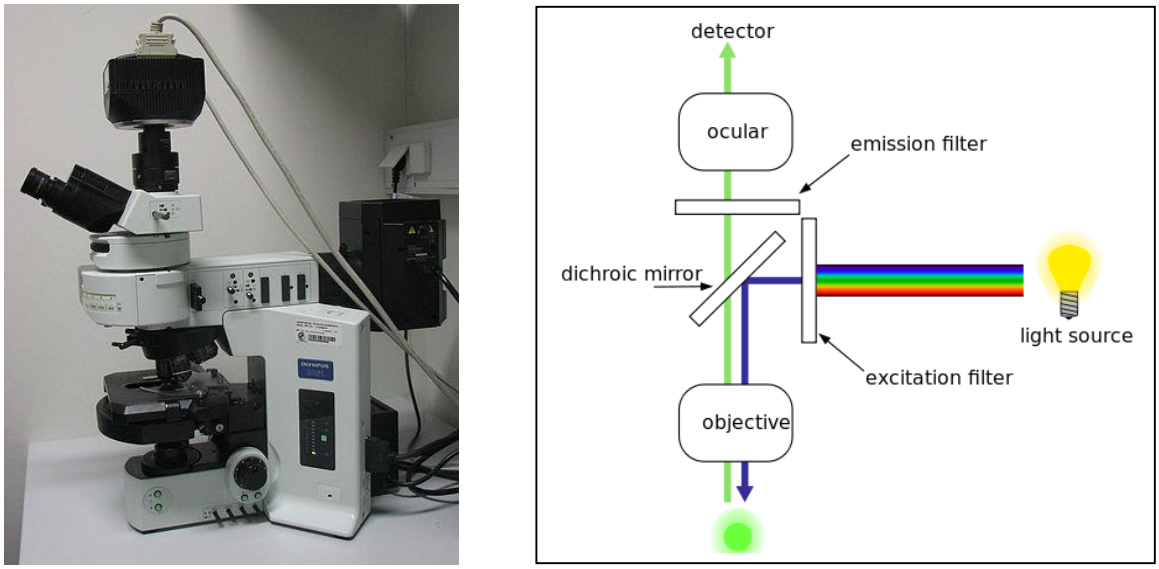


Figure:10 (A) An upright fluorescence microscope (Olympus BX61) with the fluorescent filter cube turret above the objective lenses, coupled with a digital camera. (B) Schematic of a fluorescence microscope.

4.3.2 Confocal Microscopy

Confocal microscopy is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of adding a spatial pinhole placed at the confocal plane of the lens to eliminate out-of-focus light. It enables the reconstruction of three-dimensional structures from the obtained images. This technique has gained popularity in the scientific

and industrial communities and typical applications are in life sciences, semiconductor inspection and materials science.

A laser is used to provide the excitation light (in order to get very high intensities). The laser light (blue) reflects off a dichroic mirror. From there, the laser hits two mirrors which are mounted on motors; these mirrors scan the laser across the sample. Dye in the sample fluoresces, and the emitted light (green) gets descanned by the same mirrors that are used to scan the excitation light (blue) from the laser. The emitted light passes through the dichroic and is focused onto the pinhole. The light that passes through the pinhole is measured by a detector, ie., a photomultiplier tube.So, there never is a complete image of the sample -- at any given instant, only one point of the sample is observed. The detector is attached to a computer which builds up the image.

Figure:11 The Leica TCS SP5 X confocal microscopy

5. DNA isolation by CTAB method

5.1 Materials required

5.1.1 2X CTAB BUFFER (for 10 ml)

1. NaCl – 2.8ml from 5M NaCl stock
2. Tris HCl -1 ml from 1M Tris stock

3. EDTA – 400µl from 0.5M EDTA stock
4. CTAB-0.2g

5.1.2 TE BUFFER (for 10ml)

1. 10mM Tris-100µl from 1M Tris stock
2. 1mM EDTA- 20µl from 0.5M EDTA stock

5.2 Protocol

For DNA isolation, one-day infected plant was taken.

- About 100mg tissue was taken and mixed with CTAB buffer and grinding was done in mortal pastel.
- Incubation was done at 65°C about 30 minutes and cool at room temperature.
- About 700µl chloroform was added and vortexed gently.
- Spinning was done at 12000g about 10 minutes in room temperature.
- Aqueous phase was taken.
- Isopropanol was added about 700µl and mixed well.
- Kept at room temperature and spinning at 12000g about 10 minute in room temperature.
- Supernatant was discarded.
- Ethanol (75%) was added about 500µl to pellet and spinning at 12000g for 3 minutes.
- Supernatant was discarded and pellet was air dry at room temperature about 2 minutes.
- About 20µl TE buffer was added to dried the pellet.

For visualization the DNA bands, DNA runs onto the agarose gel electrophoresis.

5.2.1 Nano drop

To quantify the amount of DNA, check the tissue sample in Nano drop

5.2.1.1 Principle

DNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the DNA purity of DNA preparation. Pure DNA has an A260/A280 of 1.8.

5.2.1.2 Procedure

Firstly, set the DNA by taking buffer as blank. Then 1-2µl of DNA sample was taken and check the concentration in µg/mL.

5.2.2 Agarose gel electrophoresis

- DNA was checked through agarose gel electrophoresis. For preparing 0.8% gel, about 40 mL of 0.5X TBE buffer (5X TAE: 54g Tris , 21.5g boric acid, 20 ml 0.5M EDTA, pH 8) was mixed with 0.32gm of agarose and then the flask was covered with a film paper to avoid loss of liquid due to evaporation and boiled in microwave.
- Then it was kept for cooling and after that 1µl EtBr was added to it.

- It was poured onto the gel casting tray (BIO-RAD) and waited for a while until it was solidified.
- Then the DNA was loaded onto the well and it was run in TBE buffer with 8V/cm.

5.2.3 After the gel was run (identified by the tracking dye, blue dye migrated upto 2/3rd of the gel length) then it was check in the gel doc (BIO-RAD).

5.2.4 DNA Fragmentation

To check the integrity of the DNA, fragmentation was done

5.2.4.1 Procedure

First of all, 1.5% of agarose gel was prepared by adding 1µl EtBr .

Then, 5µg of DNA sample was taken and 3.2µl of gel loading dye was added in it.

Sample was loaded in the well along with ladder of 1500Kb.

The sample was run 80V/cm in TAE buffer up to two-third migration.

Then, DNA bands were documented in geldoc (BIO-RAD) and visualize that DNA is degraded or not.

6. RNA isolation by LiCl precipitation

6.1 Principle

Total RNA from the 300 mg leaf tissue of *Arabidopsis* was isolated by LiCl precipitation method .The plant tissue was grind in liquid nitrogen to fine powder and was mixed with buffer A/phenol in the ratio 1:3.

6.2 Materials Required

Buffer A: Phenol(10mL):

Requirement:
8M LiCl :- 125µl
0.5M EDTA :- 200µl
20% SDS:- 500 µl
1M Tris pH9:- 1000 µl
DEPC treated water:- 8.175ml
Phenol:- 10ml

8M LiCl (125µl), 0.5M EDTA (200µl), 20% SDS (500 µl) and 1M Tris pH9(1000 µl) was added one by one in a falcon tube then maintained the volume by adding DEPC treated water. After that equal volume of phenol was added in it. Before using it should be kept in the water bath at 80°C.

6.3 Protocol

For RNA isolation, one day infected leaves sample was used.

- About 300mg plant tissue was taken and grind in liquid nitrogen.
- Powdered tissue was mixed with about 1ml buffer A: Phenol which is highly heated at 80°C.
- Vertex was done about 5 minutes.
- About 500 µl chloroform was added and vertex about 5 minutes.
- Spinning was done at 12000g about 10 minutes.
- Aqueous phase was transferred to a fresh tube.
- About 500µl chloroform was added and spinned at 12000g about 5 minutes.
- Aqueous phase was transferred into a fresh tube.

- About 500µl 4M LiCl was added to the solution and vertex was done about 3 minutes.
- The tube was incubating overnight at -20°C.
- After overnight incubation spinning was done at 14000g about 20 minute in 4°C.
- Supernatant was discarded and pellet was resuspened in 300µl TE Buffer.
- Ethanol (100%) and NaOAc (3M) were added about 750µl and 30µl respectively.
- Incubation was done about 45 minutes at -20°C.
- After incubation spinning was done at 14000g about 20 minutes in 4°C.
- Supernatant was discarded and about 500µl ethanol (70%) was added to pellet.
- Spinning was done about 14000g about 10 minute in 4°C and supernatant was discarded.
- Pellet was resuspended with 20µl DEPC water and store at -80°C for future use.

In order to visualize the RNA band, agarose gel electrophoresis was done.

6.4 Nano drop

To quantify the amount of RNA, check the tissue sample in Nano drop

6.4.1 Principle

RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the RNA purity of an RNA preparation. Pure RNA has an A260/A280 of 2.0.

6.4.2 Procedure

Firstly, set the RNA by taking buffer as blank. Then 1-2µl of RNA sample was taken and check the concentration in µg/mL.

6.5 Agarose gel electrophoresis

- RNA was checked through agarose gel electrophoresis. For preparing a 1.2% gel, about 50 mL of 0.5X TBE buffer (5X TBE: 54g Tris , 21.5g boric acid, 20 ml 0.5M EDTA, pH 8) was mixed with 0.6gm of agarose and then the flask was covered with a film paper to avoid loss of liquid due to evaporation and boiled in microwave.
- Then it was kept for cooling and after that 2µl EtBr was added to it.
- It was poured onto the gel casting tray (BIO-RAD) and waited for a while until it was solidified.

- Then about 8-10 µl RNA was mixed with 2 µl bromophenol blue and was loaded onto the well and it was run in TBE buffer with 8V/cm.
- After the gel was run (identified by the tracking dye, blue dye migrated upto 2/3rd of the gel length) then it check in the gel doc (BIO-RAD). RNA bands were documented in geldoc (BIO-RAD).

DNase TREATMENT:

- About 20µl RNA was taken.
- About 7µl DNase Buffer (10X) and 1µl DNase were added to RNA.
- Incubation was done at 37°C for 30 minutes and added DEPC water to maintained the final volume about 200µl.
- About 200µl phenol : chloroform (1:1) was added and vertexed.
- Spinning was done at 12000g for 10 minutes.
- Upper aqueous phase was transferred to fresh tubes.
- Choloform was added about 200µl and vertexed.
- Spinning was done at 12000g for 5 minutes.
- Aqueous phase was transferred to fresh tubes.
- About 0.1 volume of NaOAc and 2.5 volume of ethanol (100%).
- Incubation was done for overnight at -20°C.
- Spinning was done at 12000g for 10 minutes and supernatant was discarded.

PRIMER:

Table3: The list of primer used in the PCR.

Oligo name	Len	MW	Tm	Mg/OD	OD	µg	nmol	2ndry	GC%	Ml for 100 µm	Seq
UBQ10F	22	6725	63.7	31.8	18.8	599.5	89.1	Very weak	54.4	891	GGCCTTGTATAATCCCTGATGA
UBQ10R	22	6868	60.5	27.5	17.3	476.5	69.3	none	36.3	693	AAAGAGATAACAGGACGGAAA
EF-1aF	22	6643	67.9	35.4	9.4	333.5	50.2	moderate	50	502	TGAGCACGCTCTTCTTGCTTTC
EF-1aR	22	6772	67.8	32.6	14.8	482.8	71.2	weak	50	712	GGTGGTGGCATCCATCTTGTTA

FRK1F	19	5871	59.9	29.8	8.7	259.5	44.2	none	52.6	442	GCCAACGGAGACATTAGAG
FRK1R	20	6006	59.6	32.0	12.2	391.4	65.1	none	50	651	CCATAACGACCTGACTCATC
NHL10F	20	5997	63.6	32.8	21.8	716.7	119.5	none	50	1195	TTCCTGTCCGTAACCCAAAC
NHL10R	20	6118	63.7	32.1	17.5	562.3	91.9	weak	60	919	CCCTCGTAGTAGGCATGAGC
CYP81F2F	22	6835	63.0	28.7	14.2	407.9	59.6	none	40.9	596	AAATGGAGAGAGCAACACAATG
CYP81F2R	20	6012	63.4	32.3	14.7	475.1	79.0	Very weak	45	790	ATCGCCCATTCCAATGTTAC
PR1F	22	6825	67.9	31.2	14.2	443.1	64.9	none	54.5	649	AAAACTTAGCCTGGGGTAGCGG
PR1R	24	7199	66.2	33.6	15.3	514.4	71.4	none	45.8	714	CCACCATTGTTACACCTCACTTTG
PDF1.2aF	22	6858	66.7	29.8	11.7	348.8	50.8	Very weak	50	508	AGAAGTTGTGCGAGAAGCCAAG
PDF1.2aR	23	7160	66.8	31.5	13.3	419.8	58.6	Very weak	52.1	586	GTGTGCTGGGAAGACATAGTTGC

cDNA preparation

First of all, 18µl of template RNA was mixed in 1µl primer

Then, RNA sample was incubated on 70°C for 2 minutes

After that, sample was placed in Ice for 2 minutes

10µL of buffer plus 5µl of dNTP was added in RNA sample

Then 1µl of Reverse Transcriptase was added in it

Then, 15µl of DEPC treated water was added in the RNA sample and mixed it.

Then the sample was run on PCR.

Table4: cDNA Protocol

Reaction volume 50µl	Time
25°C	10 min
37°C	1: 30:20
75°C	15 min
10°C	∞

After this we got the cDNA, then we run the sample in normal PCR

Table5 PCR cycle programme

PCR (Vol- 10µl)	Time	
94°C	3 min	
94°C	30 sec	
55°C	20 sec	34 cycle
72°C	45 sec	
72°C	10 min	
4°C	∞	

After completing the PCR, cast 2% agarose gel in 1X TAE buffer, load the sample and run. The gel should run upto two-third. Then observed the band in UV trans-illuminator.

Result & Discussion

Three leave were taken and placed on autoclavable plate on moistened Whatman paper for pre-infection and post-infection. The scoring was done at 1dpi and are presented in the table 6 and graph1

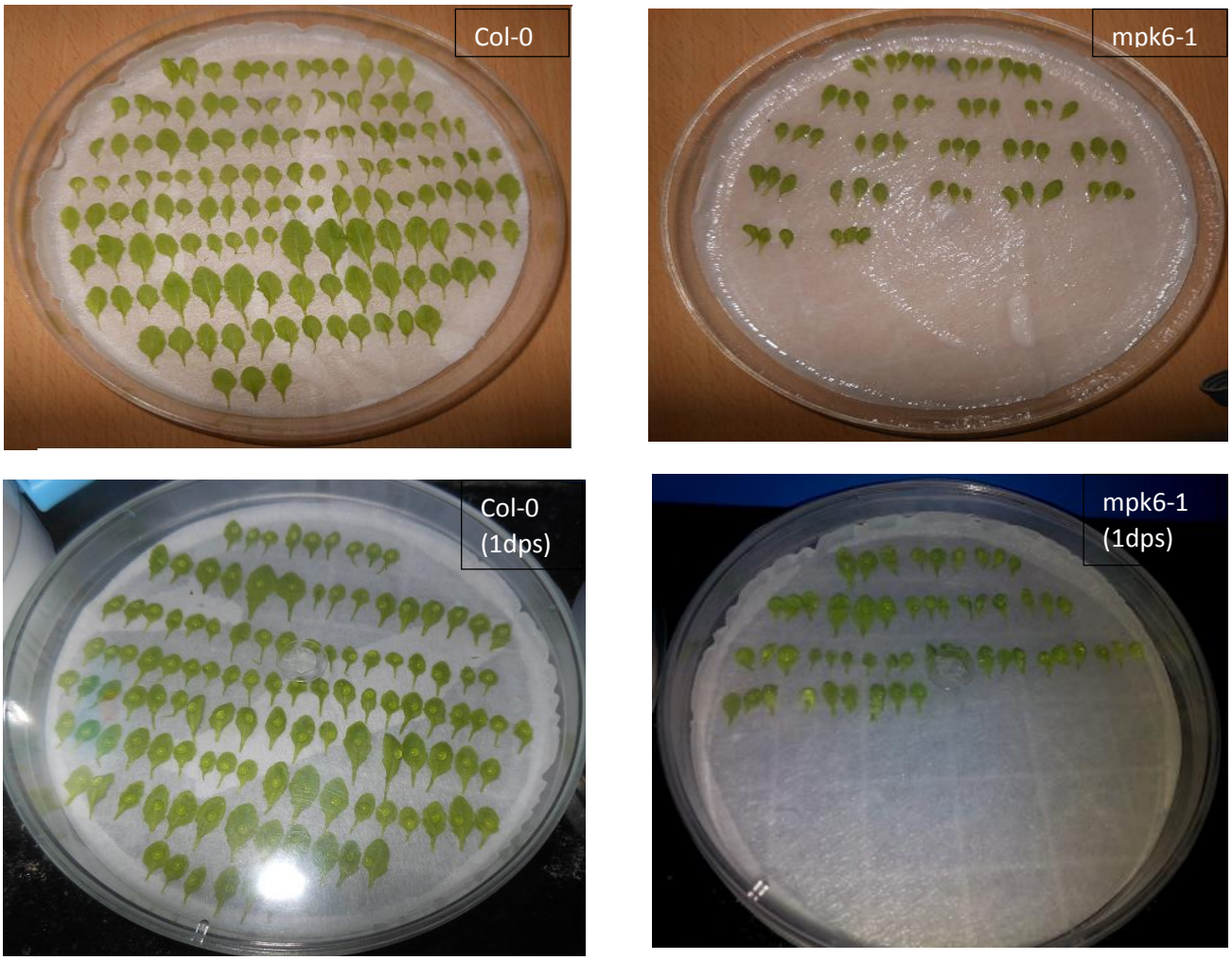


***Fig12*: 3**-4 weeks old plant with pre-infection and post-infection in Col-0 and mpk6-1

A) Phenotypic result shows after infection on leaves surface.

TABLE-6 (COL0 PLANT)

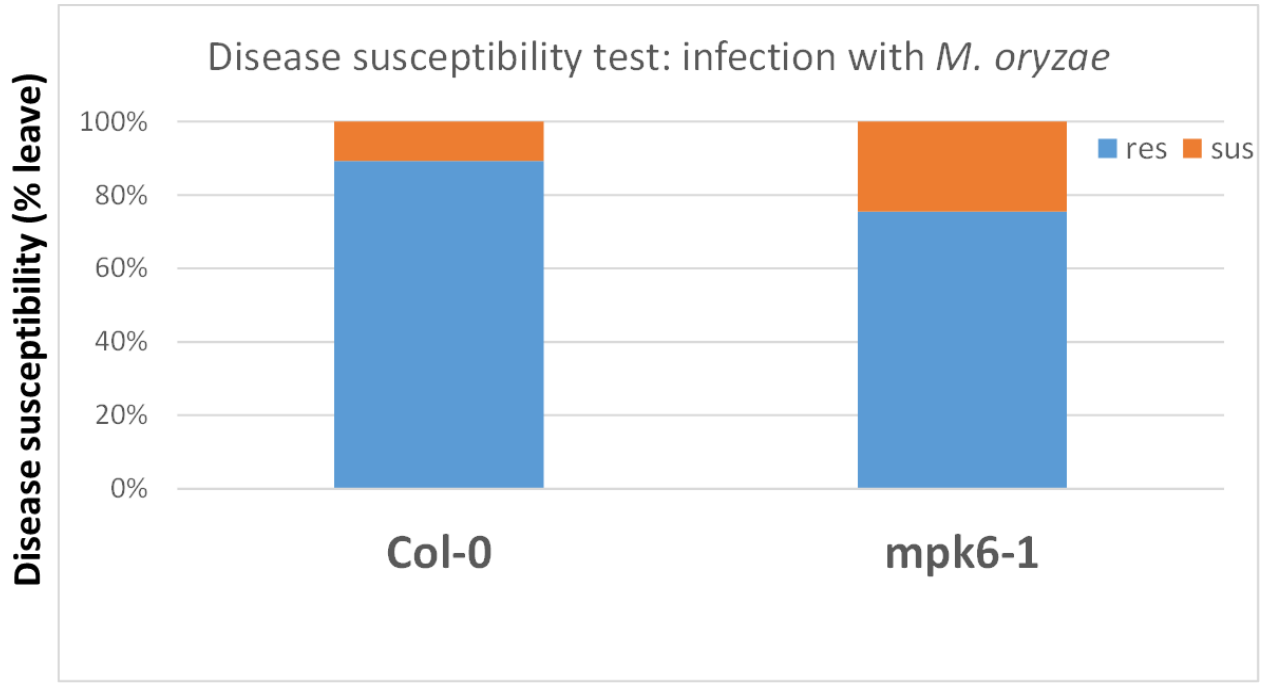
RRR	RRR	RRR	RRR
RRR	SSS	RRR	RRR
RRR	RRS	RRS	RRS
RSS	RRR	RRS	RRR
RRS	RRR	RRS	RSS
RRR	RRR	RRR	RRR

RRS	RRR	RRR	RRR
RRR	SSS	RRR	RRR
RRR	RRR	RRR	RRR
RRR	RRS	RRR	RRR
RRR	RRR	RRR	RRR
RRR	RRR	RRR	RRR
RRR	RRR	RRR	RRR
RRR	RRR	RRR	RRR
RRR			

Table 7: mpk 6-1

RRR	RRR	RRR	RRS
R_ _	RRS	S_ _	RS_
RRS	RR_	RSS	RRS
RRR	RRS	RR_	RRS
RRR	RSS	RRS	RRR

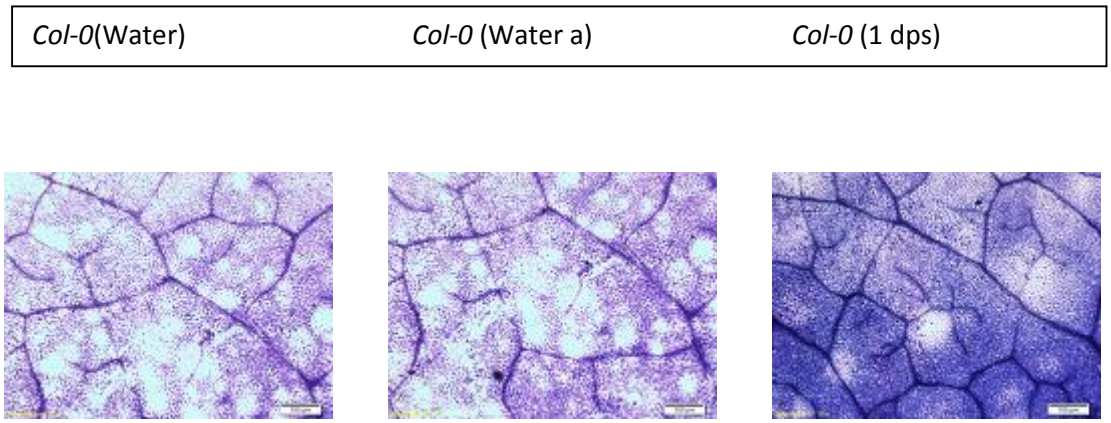
Graph 1: Graph represents the number of infected or susceptible leaf



Fluorescence microscopy of *Col-0* and *mpk 6-1* treated with water and infection

Fluorescence microscopy of Col-0 and mpk 6-1 treated with water and infection were performed in order to access the extent of mycleial growth and penetration at the infection sites (Fig 13). Mpk6-1 showed extensive infection structures as compared to the wild type Col-0 and it denotes that the mutation compromised the NHR in Arabidopsis.

(A) *Col-0*



(B) *mpk6-1*

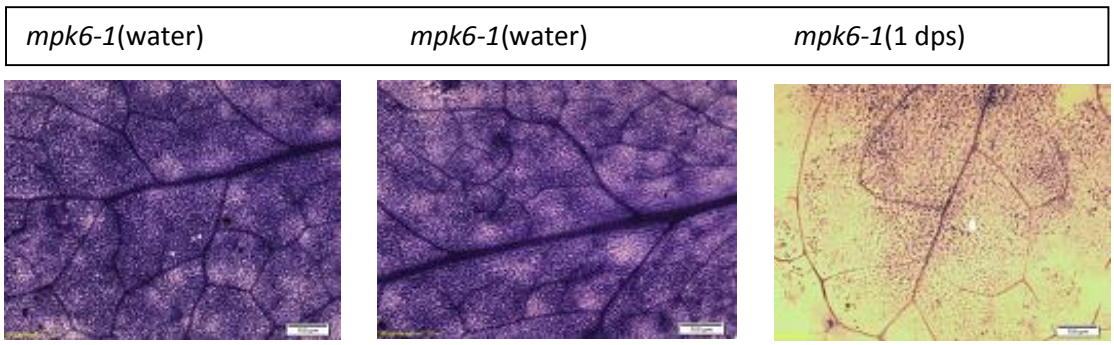


Figure 13 Fluorescence microscopy

Trypan blue stained the dead cell. Here we have *Col-0* and *mpk6-1*, water treated one day post infection. Both the samples were saw under the fluorescence microscope to observe the rate of infection. But due to low magnification fluorescence microscope, infections are not clearly visible. Further confocal microscopy was done.

Further, dual staining of the sample at 1dpi were used to take images in confocal microscope (Leica, at Dept of Biotechnology, NIT, Rourkela, facility) and are presented in Fig. 14 and 15. Extensive growth and penetration of the mycelia is visible.

To visualize the infection in wild type and mutant by Trypan-Aniline staining

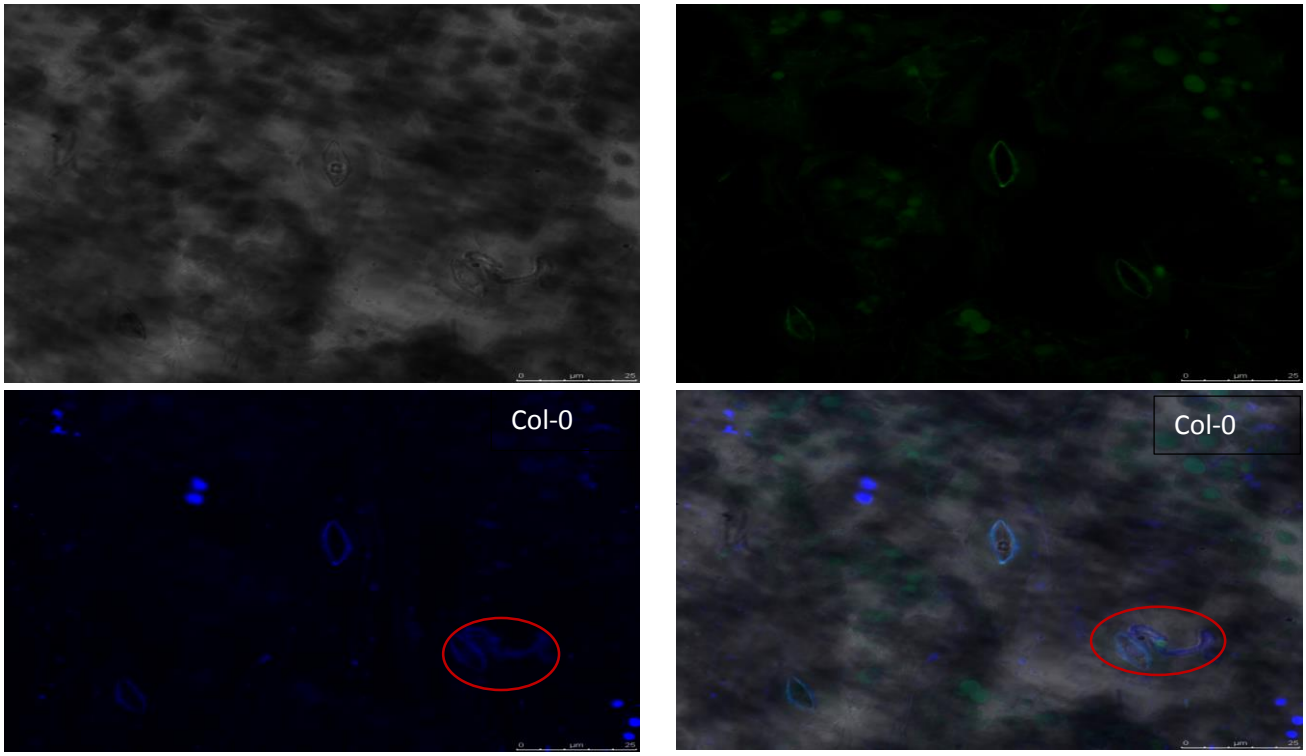


Figure14: Confocal microscopy images of Col-0 represents fungal growth

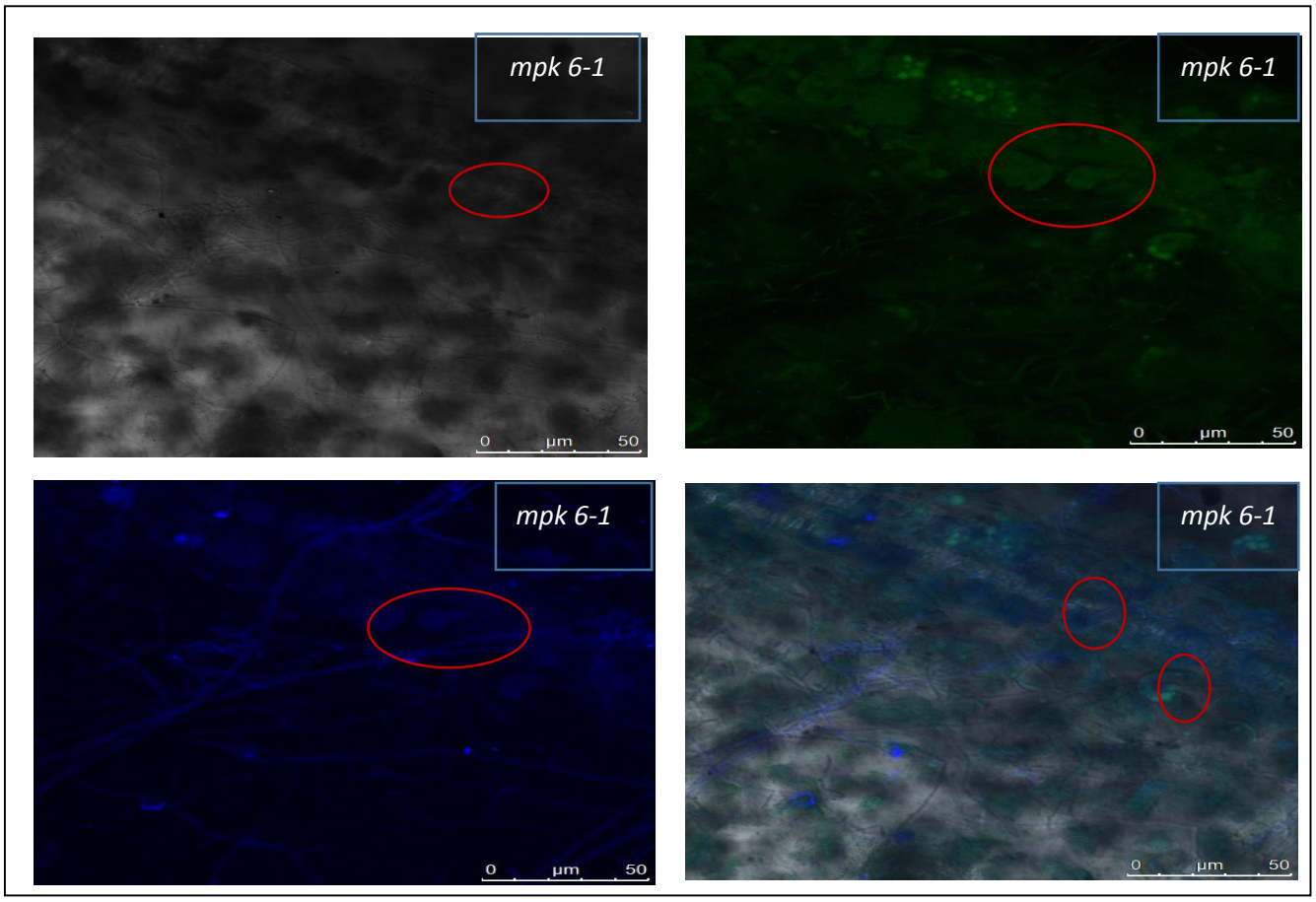


Figure 15: Confocal microscopy image of *mpk 6-1* shows extensive growth

Images of confocal microscopy after infection

A) DIC image B) aniline staining C) trypan staining D) merged image

The red circles are shown the dead cell after the infection. In Col-0 pathogen tries to enter in the leaf epidermis. There is some place in Col-0 where no infection occur.

But in case of mpk6-1 pathogen extensively enters in the leaf epidermis.

3D-image of *mpk6-1*

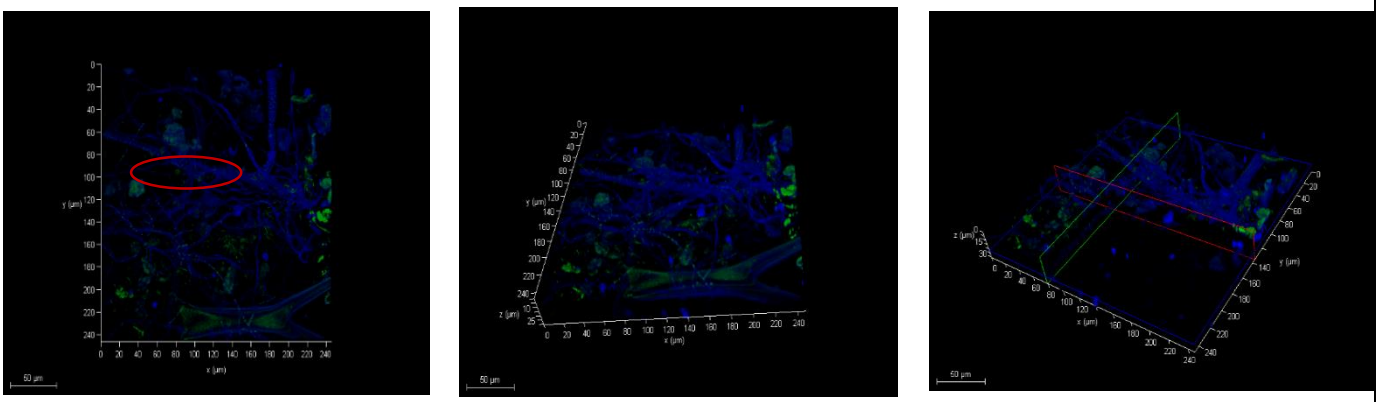
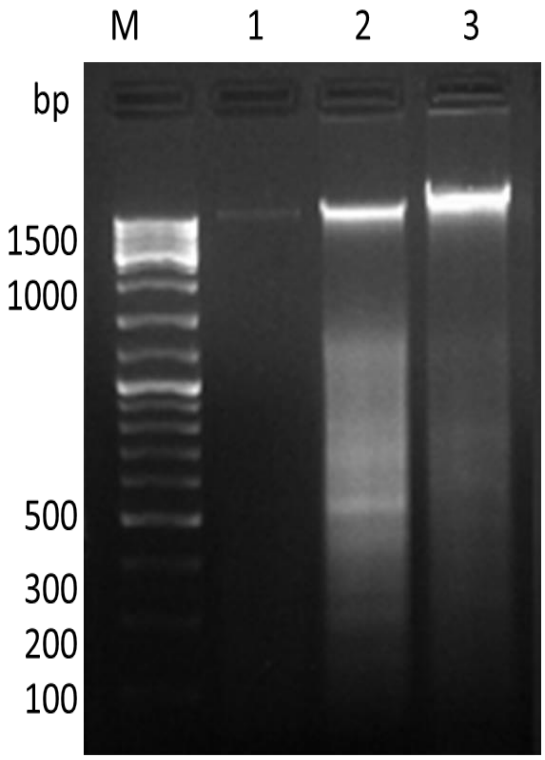


Figure16: 3DImage of mpk6-1 represents callose formation

Curiously, the 3D image was taken to see the extent of infection in the leaf samples and is presented in Fig. 16. In this 22µm macroscopic structure Pathogen extensively entering in the epidermal cell. 3D image shows the depth of the infection.

DNA FRAGMENTATION



M- Ladder (100bp-...bp)
1 - Control
2 –Col-0, infected, 1 d
3 – Mpk6-1, infected, 1 d

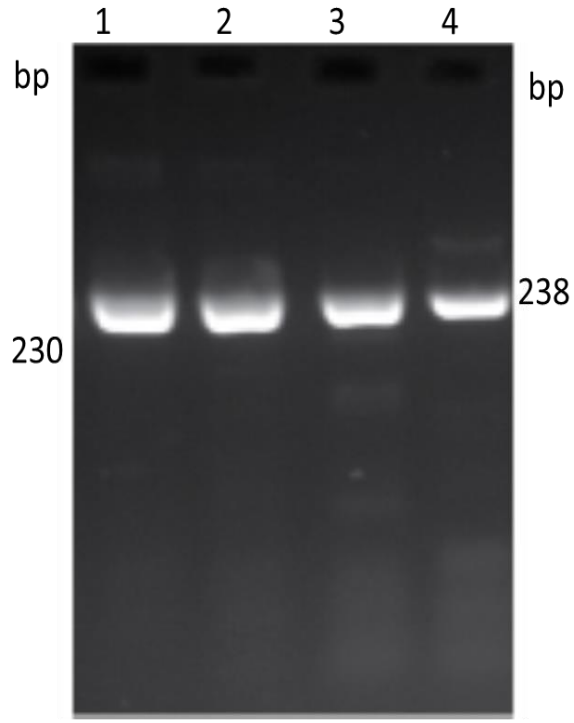
gDNA fragmentation of samples infected with *M. oryzae* Figure 16

Though mpk6 was severely infected as seen in microscopy, the gDNA is not degraded (Fig.16). Hence, the killing of cells during susceptibility might be due to some other reasons.

Defence gene signaling

In order to study the expression pattern of the defense signaling pathway genes (markers of biotrophic pathogen), we performed semi-quantitative RT-PCR (Fig. 17)

Figure 17 PCR BANDS via cDNA method Figure 17 RT-PCR of Arabidopsis samples at 1dpi. *UBQ* (PCR amplicon, 230bp) was taken as an internal control.



cDNA samples with or without treated with M. oryzae

1- mpk6-1 treated with water by adding Ubq
2- mpk6-1 treated with infection by adding Ubq
3- mpk6-1 treated with infection by adding PR1 gene
4- mpk6-1 treated with water by adding PR1 gene

Band size of sample 1 and 2 are same due to presence of Ubiquitin gene.
Band size of sample 3 and 4 are different due to the presence of PR1 gene

Conclusion

- *mpk6-1* mutant is infected more and the gene is mostly responsible for NHR, PR1 (which generally express high biotrophic pathogen) did not show any high change in expression pattern.
- So the pathogen might be hemi biotrophic as the samples tested were
- already ate (as seen from microscopy)

Future work plan

The mutant gene which provides the nonhost resistance to *Arabidopsis thaliana* was identified. Further we applied the same concept for rice plant. If the resistance gene can be transferred to the rice, then the rice may be shows resistance against the M. oryzae. The percentage of rice blast disease will be decreases and it will be helpful for the farmer and more healthy cultivation will be done. Further, the new rice germplasm will provide broad spectrum and durable resistance against rice blast and will be eco-friendly.

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